Appendix

HPLC Links:
HPLC Index

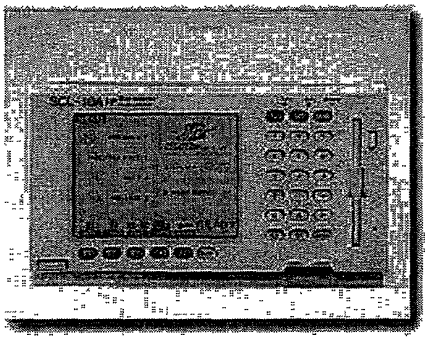
Components
SIL-HT Autosampler
Controller
Pumps
Injection/Sample Prep

Detection
Column Ovens
Accessories
Columns
Solvent Delivery
Options
Data Systems

Systems
LC-2010
VP Series
Analytical to Prep
BioInert HPLC
Drug Discovery

Reference Tools
Maintenance & Troubleshooting CD
Training Pro Software

Leasing Options
Shimadzu Leasing



SCL-10AVP

System Controller for Single Point System Control

Shimadzu's new SCL-10A*VP* System Controller directly controls all VP Series components from it's new, simplified display menu for easy, user-friendly operation. All Shimadzu HPLC system modules are conveniently linked to the controller by fiber optic cables for swift "Plug-and-Play" set-up. Fiber optic interfacing enables low noise, high-speed data transmission which enhances system reliability and sensitivity. An optional SCSI interface allows multiple VP Series HPLC systems to be linked. Pair the SCL-10A*VP* with Shimadzu's powerful CLASS-VP™ chromatography software for unmatched control capability.

New User-Friendly Interface • Large, East-To-Read Characters and Icons • Customizable Display Menu and Start-Up Screen. • On-Screen "Help" • Graphical User Interface	The new controller features many improvements including a larger, more legible display and customizable menus which simplify system operation for novice and advanced users. Menus can now be tailored to see only the parameters that you care about. Function keys, detailed help information (with valid parameter ranges), and a graphical user interface streamline parameter setup and instrument operation.
GLP/GMP	Built-in validation support

Compliance	functions help you comply with GLP/GMP regulations and enhance productivity by reducing the labor required to perform validation tasks. A record of each module's operation is preserved and can be reviewed on-screen.
Transfer and Store Methods	Save method parameters to the built-in 3.5" disk drive for reference and for secure transfer to other VP Series HPLC systems. ROM version updates are downloaded from disk for easy upgradeability.
Simple Mode for Routine Analysis	Operate your isocratic system with a simplified parameter setup. Enter pump flow rate, detector wavelength, column oven temperature and autoinjector batch schedule parameters, and begin your run.
Advanced Mode for Full Control	The SCL-10A*VP*'s advanced mode accesses full VP Series capability, including the autoinjector's automated advanced sample pretreatment functionality. All operational parameters are controlled by the SCL-10A*VP*.
CLASS-VP™ Workstation Interface	Digital signals from VP Series detectors are rapidly transmitted from the SCL-10A*VP* to the CLASS-VP™ Chromatography Workstation by standard RS-232C interface. Analog signals from other manufacturers' detectors can be converted to digital by the SCL-10A*VP* (option). Recently acquired data is saved in the SCL-10A*VP* buffer, protecting against accidental PC failure.

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Home

Products Information Service/Support Training News

HPLC Links:
HPLC Index

Components
SIL-HT Autosampler
Controller
Pumps
Injection/Sample Prep

Detection
Column Ovens
Accessories
Columns
Solvent Delivery
Options
Data Systems

Systems
LC-2010
VP Series
Analytical to Prep
BioInert HPLC
Drug Discovery

Reference Tools
Maintenance & Troubleshooting CD
Training Pro Software

Leasing Options
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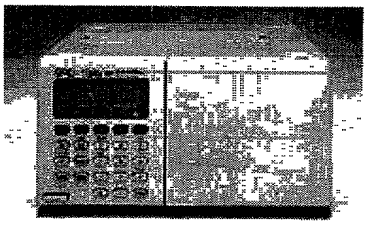

SPD-10A/10AVVP

High Sensitivity UV-Vis Detectors

The new SPD-10A*VP* and SPD-10AV*VP* VP Series UV and UV-Vis detectors combine ease of use and high sensitivity. Their optical design provides exceptionally low baseline noise, for unmatched performance. The SPD-10A*VP* and SPD-10AV*VP* deliver superb wavelength reproducibility, for high chromatographic stability. Wavelength programming optimizes component detection, and wavelength scanning allows absorbance spectra and lambda max determination. Maintenance is facilitated with front access to the flow cell and prealigned D_2 lamp which delivers double the lifetime (~2,000 hours). Simultaneous dual wavelength detection provides peak purity via ratio chromatogram for qualitative information from a single analytical run.

Unmatched Noise Specification ($\pm$0.35 x 10^{-5} AU)	Shimadzu's proven optical system delivers high sensitivity detection. The SPD-10A*VP* and SPD-10AV*VP* can detect low concentration peaks as well as peaks previously hidden in baseline noise - peaks that other detectors miss.
Baseline Stability for Reliable Analytical Results	Isolation of the light source compartment and of the monochromator reduces temperature variation, minimizing drift and reducing baseline noise.
Excellent Wavelength Reproducibility Ensures High Reliability	The high-precision monochromator grating drive mechanism has a high-resolution step motor and backlash-free force transmission system to give

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	transmission system to give exceptional wavelength reproducibility (±0.1 nm). Stability is guaranteed with a wavelength accuracy specification of ±1 nm.
GLP/GMP Compliance	Built-in validation support functions help you comply with GLP/GMP regulations and enhance productivity by reducing the labor required to perform validation tasks. A record of each module's operation is preserved and can be reviewed on-screen.
Broad Wavelength Measurement Range	The SPD-10A*VP* uses a deuterium lamp for analysis in the wavelength range of 190 - 600 nm. The SPD-10AV*VP* measures in the 190 - 900 nm range and is equipped with both deuterium and tungsten lamps for maximum sensitivity in the visible region.
Simultaneous Dual-Wavelength Measurement	The SPD-10A*VP* and SPD-10AV*VP* can acquire data at two different wavelengths in a single analytical run. The ratio chromatogram output signal delivers qualitative information on peak purity, improving analysis reliability.
Wavelength Programming Exhibits Power in Multi-Component Analysis	Using the time program function, the optimum detection wavelength for each component peak can be used automatically in one run. Analysis of multi-components with different maximum wavelengths can be performed with higher sensitivity.
Qualitative Assurance by Wavelength Scanning	The wavelength scanning function creates an absorbance spectrum of the target component using the "stopped flow" technique. Since the background spectrum of the mobile phase can be subtracted, true spectrum are

	subtracted, true spectrum are attained.
Long-Life Deuterium Lamp	The new deuterium lamp for the SPD-10A*VP* and SPD-10AV*VP* lasts twice as long as before. Program the lamps to turn on and off automatically, further increasing useful lamp operation time.
Pre-Aligned Lamp Provides Easy Installation	The design of the SPD-10A*VP* and SPD-10AV*VP* enables fast, user-friendly lamp installation without time-consuming adjustments. The enhanced life of the deuterium lamp and new, pre-aligned lamp assembly design reduce detector maintenance.
Wide Array of Flow Cell Options	Optional flow cells for the SPD-10A*VP* and SPD-10AV*VP* support both micro and preparative applications. The cells of the SPD-10A*VP* and SPD-10AV*VP* are accessible from the front of the detector, for faster and easier maintenance. You can reconfigure the instrument for semi-micro HPLC without modifying plumbing in the existing HPLC system.
Solvent Recycle Kit	The optional solvent recycle valve kit decreases isocratic HPLC mobile phase consumption by recycling clean discharge. A detector threshold setting determines whether solvent is sent to the waste container or mobile phase reservoir. This kit reduces operating costs and benefits the environment.

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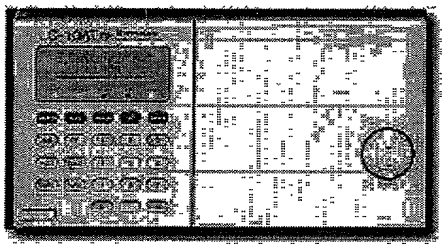

LC-10ADvp/LC-10ATvp

Solvent Delivery Units for Shimadzu VP Series HPLC System

The VP Series offers two solvent delivery units. Choose the one that's right for your HPLC application. Shimadzu pumps have built-in validation functions for GLP/GMP compliance, and offer front access to seals and plungers for fast maintenance. New, improved seal and plunger materials provide increased pump durability, and leak sensors are standard for added safety. Both pumps can be used for a wide array of applications. Set them up in isocratic, high-pressure gradient, or low-pressure gradient configurations.

The **LC-10ADvp** micro-plunger pump is unsurpassed for stable, pulse-free solvent delivery. It is perfect for low flow rate semi-micro and LC/MS applications as well as for high sensitivity analyses.

The **LC-10ATvp** serial dual plunger pump is extremely durable. It is well-suited for routine analyses and low pressure gradient operations.

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Multiposition

High pressure

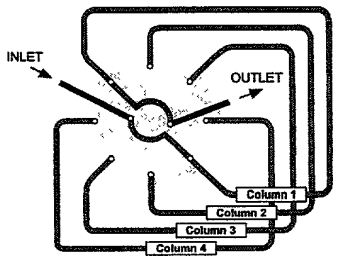
ST

1/16"

0.40 mm

Both column ends selected – ST configuration

ST valves are used for multi-column, multi-sample, or multi-trap operations. This valve can be used between an injector and detector to permit manual or automated HPLC column selection. For an application suggestion, see page 161.

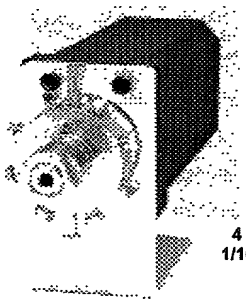


1/16" fittings, 0.40 mm ports (.016")

UW Type

Standard electric actuators: 110 VAC for USA; 110/230 VAC to 24 VDC power supply for international.
Microelectric actuators: 24 VDC, with 110/230 VAC to 24 VDC power supply.

	4 Columns or Loops *Prod No*	6 Columns or Loops *Prod No*
Manual (not recommended)	CST4UW	CST6UW
With air actuator	ACST4UW	ACST6UW
With standard electric actuator	ECST4UW	ECST6UW
With microelectric actuator	EMTCST4UW	EMTCST6UW
Replacement valve	DCST4UW	DCST6UW
Replacement rotor	SSACST4UW	SSACST6UW



ST 4 position 1/16" fittings

1/16" Stainless steel loops *for UW Type valves*

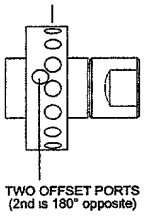
Each stainless steel loop includes two stainless nuts and two stainless ferrules. Order special fittings separately.
When a set of loops is ordered, loops will be supplied from the same lot.

Volume	*Prod No*	*Volume*	*Prod No*
10 µl	SL10CSTUW	250 µl	SL250CSTUW
15 µl	SL15CSTUW	500 µl	SL500CSTUW
20 µl	SL20CSTUW	1 ml	SL1KCSTUW
25 µl	SL25CSTUW	2 ml	SL2KCSTUW
50 µl	SL50CSTUW	5 ml	SL5KCSTUW
100 µl	SL100CSTUW	10 ml	SL10KCSTUW

FURTHER REFERENCE

ABOUT LOOPS

- Other materials available in many sizes: Electroformed Nickel, Hastelloy C, Nickel 200, PEEK, PTFE, and Titanium
- Loops > 2 ml are made from 1/8" OD tubing with brazed or welded 1/16" tube ends or reducing unions.



SPECS

5000 psi liq
75°C max

Valve body:
Nitronic 60
Stainless

Rotor:
Valcon E

Trap Cartridges

Michrom BioResources, Inc. offers a variety of trap cartridges packed with application specific HPLC column packing materials for concentration, desalting, detergent removal and protein removal from samples prior to analysis by HPLC, LC/MS, MALDI-MS, Edman sequencing and/or amino acid analysis. These cartridges can be used individually or in series to cleanup samples manually, online using a HPLC injector or automatically with a HPLC autosampler. These application specific cartridges are available in three sizes: capillary (CapTrap™), microbore (MicroTrap™) and macrobore (MacroTrap™), with capacities as shown in the table below.

Cartridge Type	Bed Volume	Sample Capacity	Sample Volume	Speed of Loading
CapTrap (0.5 X 2 mm)	0.5 ul	2 ug	0.1 - 100 ul	5 - 20 ul/min
MicroTrap (1 X 8 mm)	5.0 ul	20 ug	1.0 - 1000 ul	50 - 200 ul/min
MacroTrap (3 X 8 mm)	50 ul	200 ug	10 - 10000 ul	500 - 2000 ul/min

Click on the following links to get information on the types of Trap Cartridges

Amino Acid Traps	ISRP Traps	NID Traps	Peptide Traps
Protein Traps	SCX Peptide Traps	SDS Traps	Small Molecule Traps

Manual Sample Preparation

Samples can be cleaned up without the use of a HPLC by using a manual system as shown in Figures 1 and 2.

Figure 1. Manual CapTrap Holder Kit With Micro-Macro Holder and 10-32 Male/Male Union. For Use in Two Step Applications Such as SDS Removal From Proteins and Peptides.

Figure 2. Manual Micro - Macro Trap Holder Kit

All Manual Holder Kits Include: Syringe, filler port, trap holder, and outlet tube for sample elution

Holder Description	Part Number	Syringe Size	Sample Elution Volume
Manual CapTrap Holder Kit	602/25029/03	*10 ul*	*For 1-5 µl sample elution*
Manual MicroTrap Holder Kit	004/25111/01	*25 ul*	*For 5-25 ul sample elution*
Manual MacroTrap Holder Kit	004/25111/02	*100 ul*	*For 20 -100 ul sample elution*

On-Line Sample Preparation

Samples can be cleaned up on-line using a manual or automated HPLC injector with the trap cartridge built into the loop, as shown in Figures 3 and 4. Dilute samples can easily be concentrated and then washed with the starting HPLC mobile phase to remove salts and other non volatile buffers prior to analysis by HPLC, LC/MS or FIA/MS. Michrom's trap cartridges can also be used with the MAGIC HPLC autosampler (and other autosamplers with sample preparation capabilities) to automate concentration, desalting and detergent or protein removal from a variety of biological and pharmaceutical samples.

Loop/Holder Includes: Trap holder and Two Connecting Tubes with Fittings

Holder Description	Part Number
Valco CapTrap Loop/Holder	602/25029/00
Rheodyne CapTrap Loop/Holder	602/25029/01
Valco MicroTrap Loop/Holder	004/25110/00
Rheodyne MicroTrap Loop/Holder	004/25111/00
Valco MacroTrap Loop/Holder	004/25110/00
Rheodyne MacroTrap Loop/Holder	004/25111/00

Capillary: 50u ID PEEKSIL Tubing. Total Holder, Trap & Tubing Volume is <1 ul
Micro: 0.007" ID PEEK Tubing. Total Holder, Trap & Tubing Volume is <10 ul
Macro: 0.007" ID PEEK Tubing. Total Holder, Trap & Tubing Volume is <60 ul

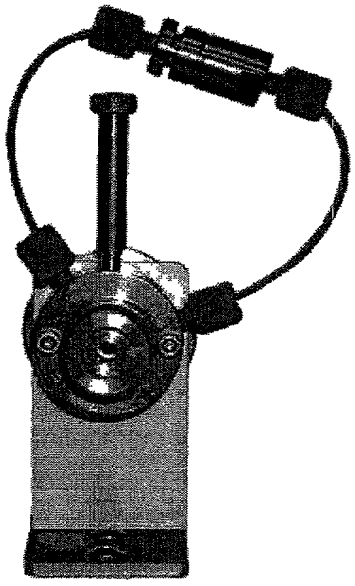


Figure 3. Valco Manual HPLC Injector With CapTrap Loop/Holder and Syringe Filler Port.

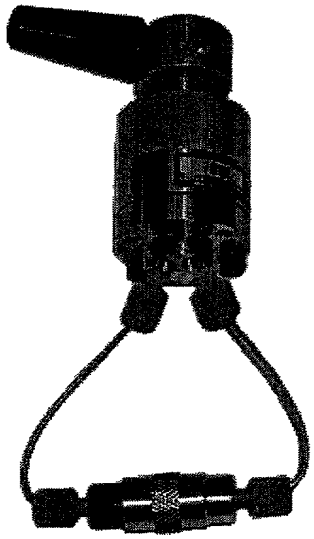
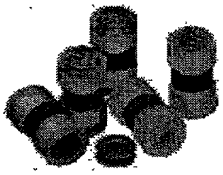
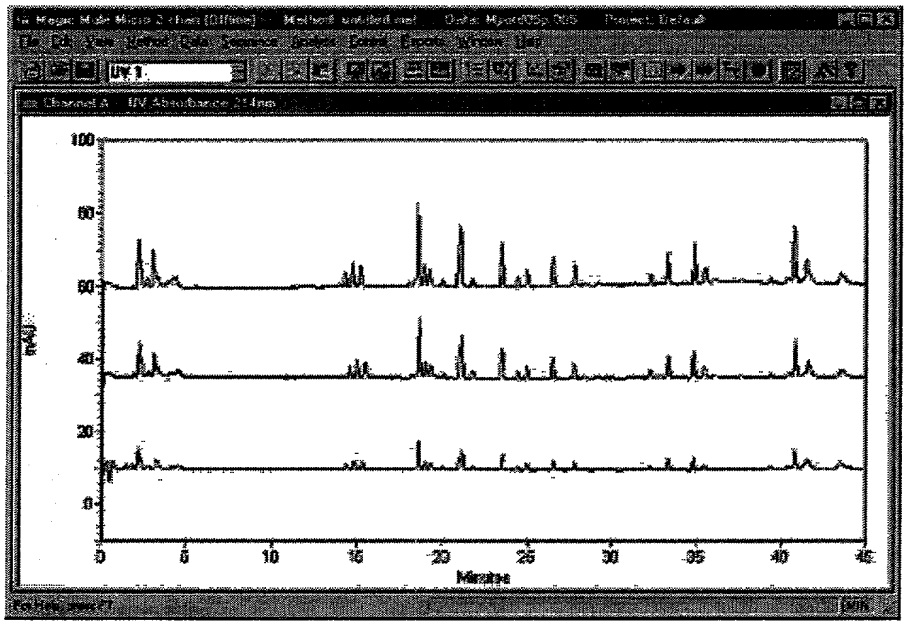


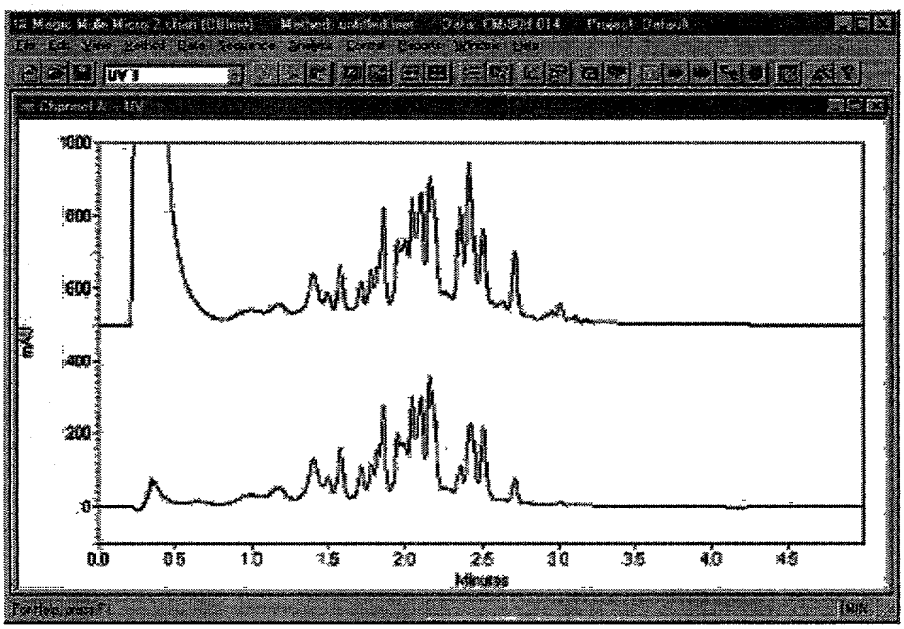
Figure 4. Rheodyne Manual HPLC Injector With Micro - Macro Trap Loop/Holder.

Peptide Concentration & Desalting Traps





On-line concentration of a dilute protein digest from a 2D gel (1 pmol/ml) on a peptide microtrap for trace LC/MS analysis



Automated desalting of recombinant protein digest on a peptide macrotrap for fast LC/MS quality control

The Michrom Peptide Trap Cartridges contain a medium pore, large particle, polymeric reversed-phase HPLC column packing that is designed to bind protein digests, peptides, small polyneucleotides and other small biological molecules (0.5 – 50 kD). The peptide trap is used to concentrate samples and remove salts, buffers and other small, polar molecules prior to analysis or characterization using HPLC, LC/MS, MALDI TOF-MS, Edman Sequencing or other techniques. The peptide trap can also be used in series with a SDS removal trap for samples that require SDS detergent removal as well as concentration and/or desalting. Michrom's peptide traps can be used to concentrate samples up to 100 fold and can effectively remove salts and nonvolatile buffers to greater than 99.9%. The polymeric packing material allows binding of moderately hydrophilic compounds and most small component biological samples in as little as 1% organic solvent, yielding excellent recovery (>90% at levels above 10 femtomoles). The Michrom Peptide Trap Cartridges can be cleaned with strong acid, strong base and/or organic solvents to eliminate carry-over and allow the trap to be used for hundreds to thousands of samples. For large hydrophobic peptides (ie CNBr digests) the protein trap may be more appropriate, while for very small hydrophilic peptides (ie phosphopeptides) the small molecule trap may be more appropriate.

Description	Band Color	Capacity	Part Number
Peptide MacroTrap (Each)	Green	200 ug	004/25108/52
Peptide MacroTrap (6 Pack)	Green	200 ug	004/25109/52
Peptide MicroTrap (Each)	Green	20 ug	004/25108/02
Peptide MicroTrap (6 Pack)	Green	20 ug	004/25109/02
Peptide CapTrap (Each)	Green	2 ug	004/25108/32
Peptide CapTrap (6 Pack)	Green	2 ug	004/25109/32

Anal. Chem. **1997**, *69*, 1518–1524

Comprehensive On-Line LC/LC/MS of Proteins

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This is a description of a comprehensive two-dimensional liquid chromatography (LC) system for the separation of protein mixtures. This system uses cation-exchange chromatography followed by reversed-phase chromatography (RPLC). The two LC systems are coupled by an eight-port valve equipped with two storage loops and under computer control. The RPLC effluent is sampled by both a UV detector and an electrospray mass spectrometer. In this way, complex mixtures of large biomolecules can be rapidly separated, desalted, and analyzed for molecular weight in less than 2 h. The system's utility is demonstrated with a mixture of standards and an *Escherichia coli* cell lysate.

Comprehensive LC/LC systems were invented to simplify the separation of proteins which overwhelm one-dimensional LC systems[1,2] or even hyphenated techniques such as CE–MS or LC–MS. These comprehensive techniques differ from ordinary heart cutting two-dimensional (2D) systems[3–9] in that they subject the entire sample to 2D separation. The two dimensions should ideally be orthogonal,[10] and any separation accomplished by the first dimension again should ideally be retained upon transfer to the second dimension. The coupled column or heart cutting systems usually work by trapping a few analytes of interest and then separating those by reversed-phase LC. All other sample components are diverted to waste. In a comprehensive system, all analytes are acted upon equally by both dimensions, without the diversion of only a few species to the second dimension. This LC-based system leaves the analytes in solution as opposed to being bound in a matrix, as does two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis[11,12] (2D-PAGE).

Until this time, the only report of coupling a comprehensive 2D separation to mass spectrometry made use of reversed-phase chromatography followed by capillary electrophoresis for the analysis of peptides.[13] Unfortunately, the physical constraints of that system make it impossible to collect analytes after they have been separated and identified. Other reports of comprehensive 2D systems used either laser-induced fluorescence for the detection of peptides[14,15] or UV exclusively for the detection of proteins.[16–19]

The system described here for the separation of protein mixtures comes close to Giddings's criteria[20] for an ideal 2D system, as it makes use of cation-exchange chromatography followed by reversed-phase chromatography, two orthogonal modes. Furthermore, since the RPLC column samples the first dimension two or three times per peak, this system tends not to recombine components already separated by the cation column. By making use of liquid chromatography, this system allows fractions to be readily collected following separation. Finally, this is the first report of comprehensive LC/LC utilizing mass spectrometry for detection, which yields on-line molecular weight information. This adds, in essence, a third dimension to this 2D system, because the mass spectrometer can identify the presence of coeluting peaks when they are not resolved by chromatography.

EXPERIMENTAL SECTION

Overview. The basic layout of the system draws on the design of Bushey and Jorgenson.[16] Figure 1 is a schematic of the new system. A pump delivers mobile phase to the injector and onto a cation-exchange column. This column's outlet is attached to a two-position, eight-port valve. As one loop fills with effluent from the ion-exchange column, the other loop is being pumped out by a second HPLC pump and through the RPLC column. This column's effluent flows through a UV detector and is split, and 10% flows to the mass spectrometer.

Liquid Chromatography. The cation-exchange chromatography begins with a dual-syringe pump (ABI 140B, Perkin Elmer,

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S0003-2700(96)01155-9 CCC: $14.00

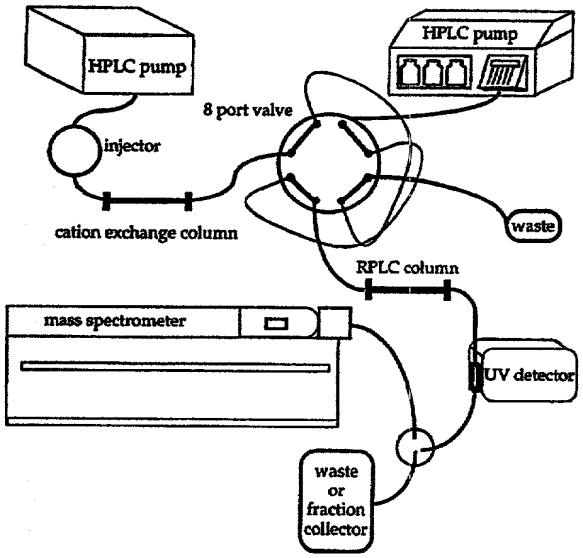

Figure 1. Schematic of LC/LC/MS instrumentation.

Norwalk, CT) delivering mobile phase to a 2.5 μL injector (Rheodyne, Cotati, CA). The column is packed in-house with Bakerbond Wide-Pore Carboxy-Sulfon 5 μm material (J.T. Baker, Phillipsburg, NJ), and its dimensions are 750 μm i.d. × 12.5 cm length. The mobile phase gradient consists of A = 50 mM sodium formate (Sigma, St. Louis, MO), 3 M urea, and 10% acetonitrile (J.T. Baker) at pH = 4, B = 1000 mM ammonium formate, 3 M urea, and 10% acetonitrile at pH = 6. A typical gradient runs from 20% B to 100% B in 2 h at 10 μL/min.

The chromatography system is controlled from a Macintosh Quadra 900 (Apple Computer, Cupertino, CA) running LabView software (National Instruments, Austin, TX) and using an NB-MIO-16XL board (National Instruments) for valve switching, triggering the mass spectrometer, and collecting UV data. The valve is an eight-port, two-position valve (EC8W, Valco Instruments, Houston, TX). Loop volume is determined by multiplying the flow rate of the ion-exchange column by the sum of the run and reequilibration times of the RPLC column.

The reversed-phase column is packed in-house with POROS R2/H material (PerSeptive Biosystems, Framingham, MA), and its dimensions are 500 μm i.d. × 10 cm length. It is supplied with mobile phase from an HP 1090 (Hewlett Packard, Palo Alto, CA), which is flow-split from 5 mL/min to 50 μL/min. Mobile phase A is water with 0.1% trifluoroacetic acid, and B is acetonitrile with 0.1% TFA. A typical gradient runs from 22% B to 100% B in 1.25 min, followed by a 0.25 min reequilibration step at initial conditions. All connections are through 62 μm i.d. × $^1/_{16}$ in. PEEK until the outlet of the RPLC column. From this outlet to the splitter, a 150 μm i.d. × 360 μm o.d. fused silica capillary is used for on-capillary UV detection (Model 200, Linear Instruments,

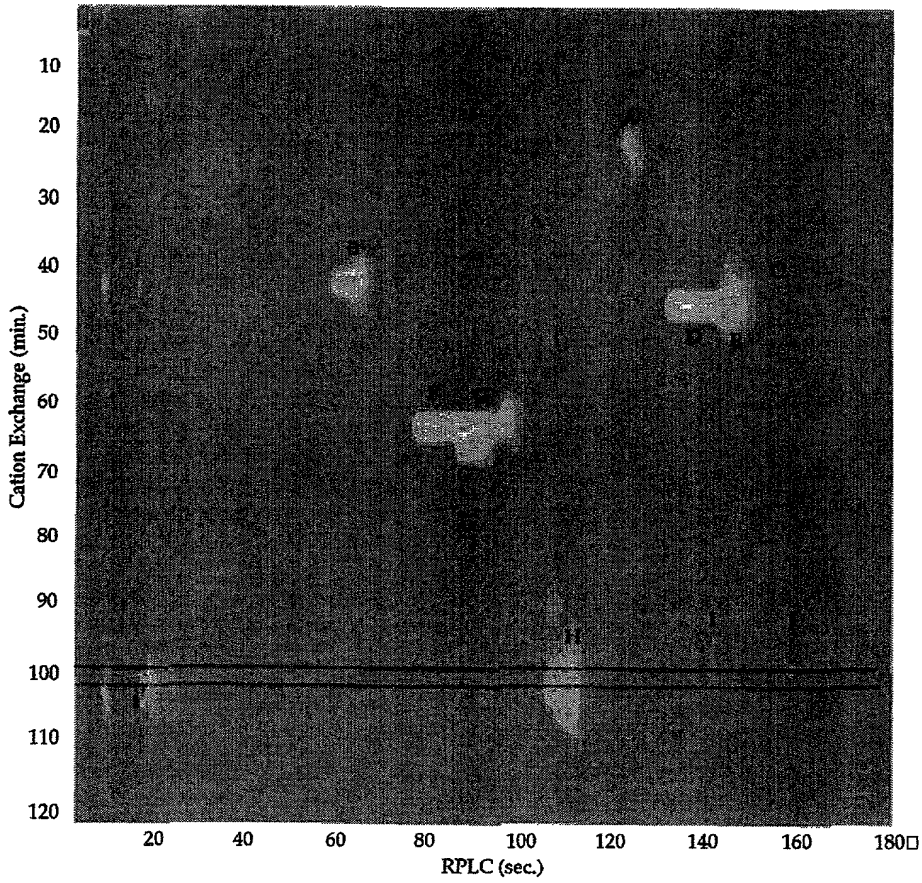

Figure 2. 2D chromatogram of protein standards: peak A, α-Lac; B, RNA; C, CAH; D, β-Lac A; E, β-Lac B; F, HH Cyt *c*; G, BH Cyt *c*; H, Lys; I, α-Hb; J, β-Hb.

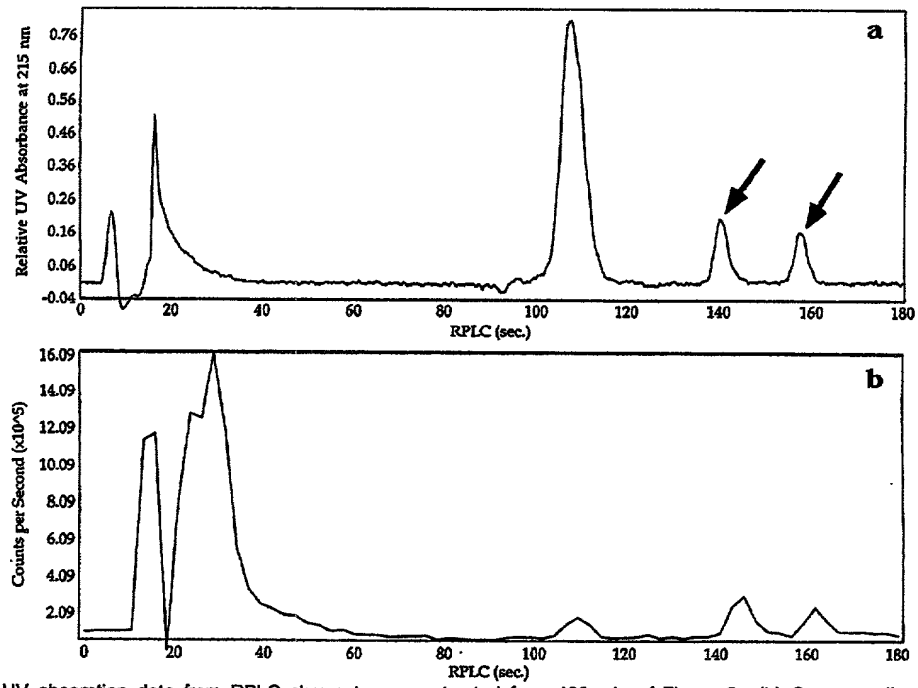

Figure 3. (a) UV absorption data from RPLC chromatogram extracted from 100 min of Figure 2. (b) Corresponding total ion current chromatogram.

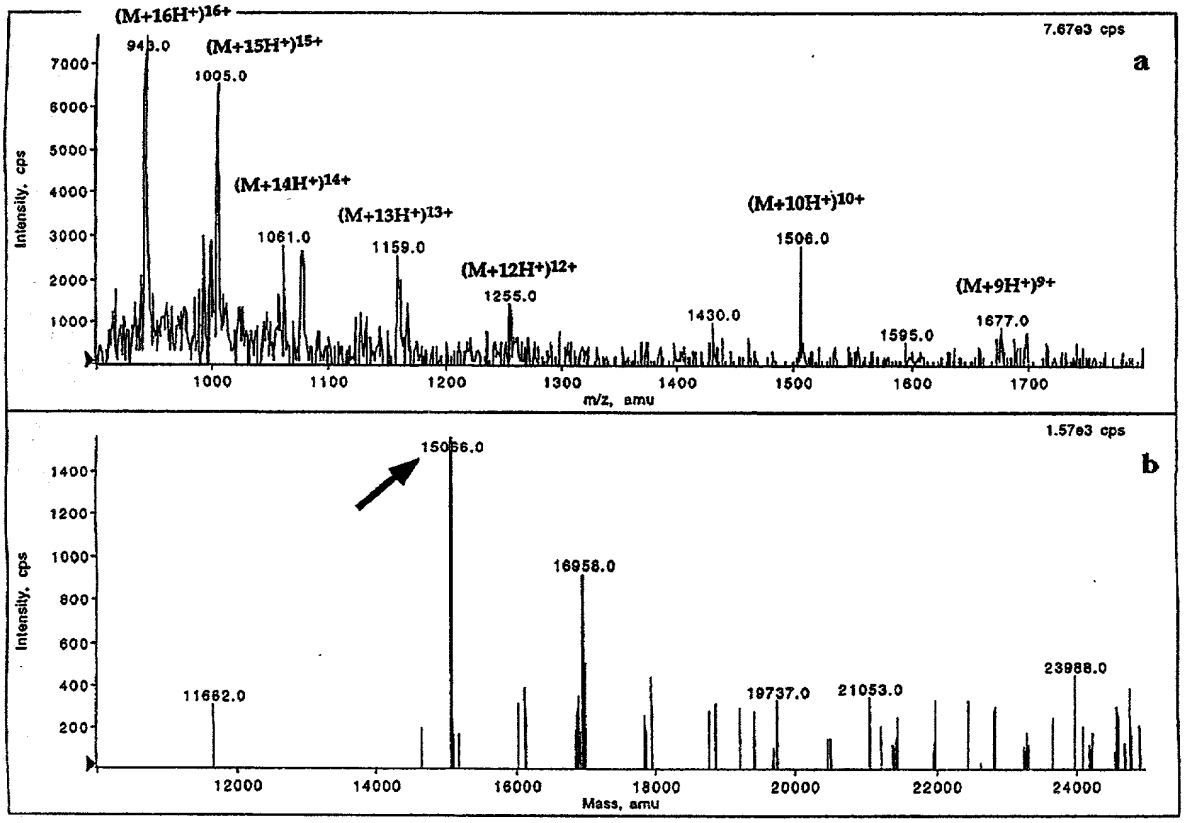

Figure 4. (a) Mass spectrum from peak at 142 s of Figure 3. (b) Corresponding Hypermass reconstruction of charge envelope.

Reno, NV). Following the splitter, a 29 μm i.d. × 150 μm o.d. capillary supplies the electrospray interface.

Mass Spectrometry. The mass spectrometer is a Sciex API 100 (Perkin Elmer) and is controlled by a Macintosh 8100 using

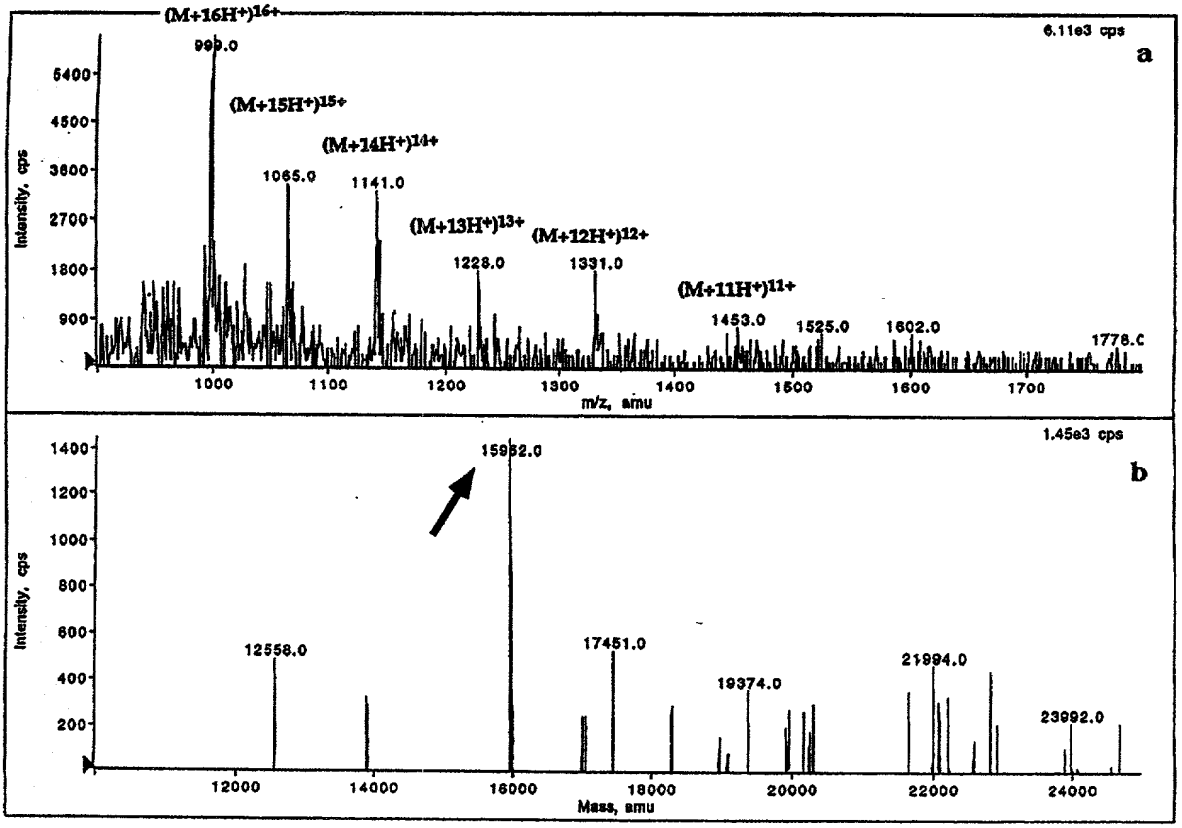

Figure 5. (a) Mass spectrum from peak at 162 s of Figure 3. (b) Corresponding Hypermass reconstruction of charge envelope.

either the supplied Sample Control or LC2Tune software. It scans from m/z 1000 to 2000 in 0.1 steps with a dwell of 0.2 ms, for a total single scan time of 2 s. If the LC2Tune program is used, the entire 2D data set is recorded as a single experiment whose duration is that of the cation-exchange run time plus one RPLC run. While this collects all data, including the salt peak and reequilibration time, it stores this data in volatile random access memory (RAM). This limits the number of data points that can be collected to the computer's RAM and is susceptible to loss in the event of a power interruption or surge. Another way to collect the mass spectral data is to use the Sample Control program, which receives a start signal at the beginning of each RPLC run and collects until 12 s from the end of that run, when it bundles the data, writes it to disk, and waits for the start of the next RPLC run. With this method, only the current RPLC run is stored in volatile memory, and 2D experiments are limited only to the size of the hard disk drive.

Data Workup. Data from the UV detector are background subtracted with a LabView program written in-house and displayed using Transform 3.0 (Fortner Research, Sterling, VA). The mass spectrometer data are analyzed in Bio-Multiview (ver. 1.0, Perkin-Elmer), which calculates the molecular weight of a chromatographic peak from its charge envelope.

Sample Preparation. Protein standards are purchased from Sigma, dissolved in IEC mobile phase A, and filtered through 0.45 μm filters (UFC3 OHV 25, Millipore, Bedford, MA). The standards used are α-lactalbumin from bovine milk (α-Lac), ribonuclease A(RNA) from bovine pancreas, β-lactoglobulins A and B from bovine milk (β-Lac A, β-Lac B), carbonic anhydrase from bovine erythrocytes (CAH), cytochrome *c* from horse heart and bovine heart (HH Cyt *c*, BH Cyt *c*), bovine hemoglobin (α-Hb, β-Hb), and lysozyme from chicken egg white (Lys). Each protein's concentration in the standard mixture is 1 mg/mL, except the β-lactoglobulins, which are 0.5 mg/mL each. The *Escherichia coli* sample comes as intact cells in the fermentation broth. The cells are centrifuged to the bottom of the tube at 6000*g* for 1 h. The broth is removed, and the cells are resuspended in an equivalent volume of IEC mobile phase A for a total protein concentration of ~7.5 mg/mL, determined by amino acid analysis. The cells are sonicated in a cold water bath for 30 min and recentrifuged. The supernatant is withdrawn and filtered through a 0.45 μm filter.

RESULTS

Figure 2 shows the separation of 10 protein standards as detected by UV absorption at 215 nm. A component's coordinates are obtained by observing its retention time on the cation-exchange column from the *y*-axis and its RPLC retention time from the *x*-axis. In this case, one 120 min cation-exchange run is sampled by 48 individual 150 s duration RPLC runs. The streak down the *y*-axis between 5 and 20 s of the RPLC runs results from the salt and urea in the first dimension. At 40 and 100 min (IEC run time), the intensity of the salt and urea peak, components which are unretained on the RPLC column, rises out of the background of the 2D plot. At these times, the simultaneous injection of a large quantity of salt, urea, and protein saturates

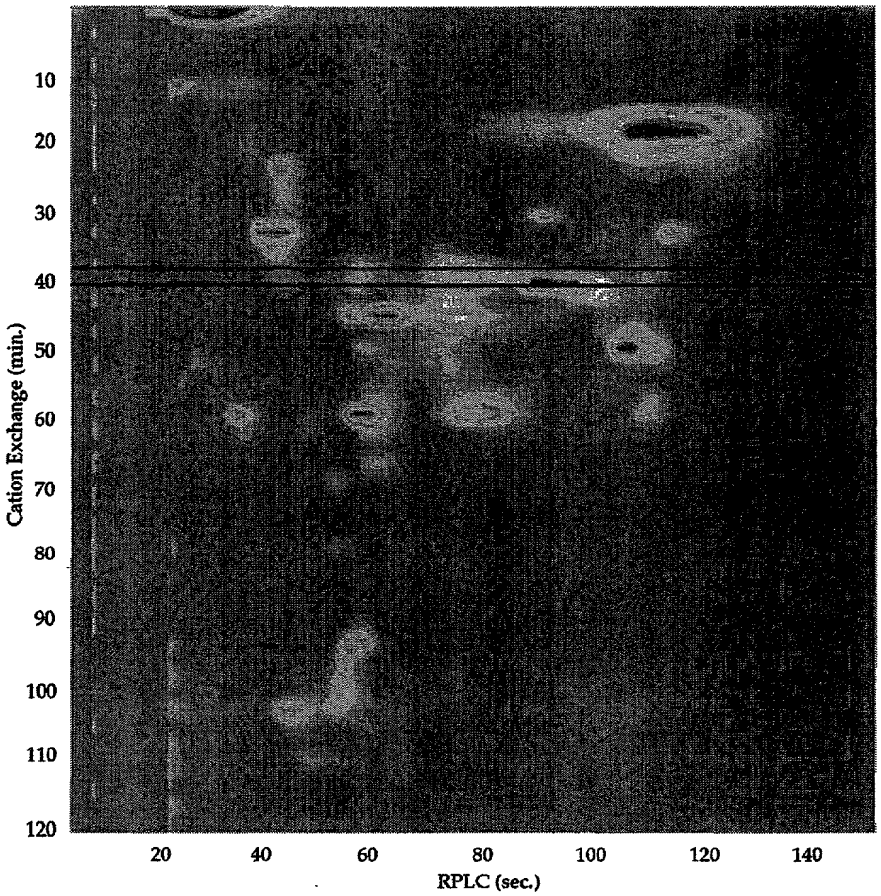

Figure 6. 2D chromatogram of *Escherichia coli* lysate.

the 750 μm i.d. RPLC column, which causes the unretained peak to tail. Since the data for the 2D plot are background subtracted using an RPLC run with only a salt and urea injection, the tailed peak creates the artifacts observed in the RPLC runs beginning at 40 and 100 min. These are the only places such an event occurs in the 2D plot because the total salt, urea, and protein loads during other RPLC runs are not sufficient to saturate the RPLC column.

The shapes of peaks in the 2D plot are examined in closer detail by extracting the individual RPLC run(s) in which they appear. For example, the individual RPLC run at 100 min of the IEC run is shown in Figure 3, where both the UV absorption and total ion intensity data are plotted. Peaks are observed at 112, 142, and 162 s in the UV and slightly later in the ion current data because of the void volume between the detectors. The peaks in the ion current appear broader than those in the UV because of the lower data acquisition rate of the mass spectrometer (0.5 vs 4 Hz). The mass spectra of the two later-eluting peaks make up Figures 4a and 5a. The Hypermass reconstruction of these peaks, shown below each spectrum in Figures 4b and 5b, identifies them as the two chains of hemoglobin. The last-eluting peak results from an injection onto the front of the system of 16 pmol of intact hemoglobin or 32 pmol of the β-chain. The entire peak flows through the UV detector, but the mass spectrometer, because it follows the 10:1 split, is presented with 3.2 pmol.

Reexamining Figure 2 shows that most peaks eluting off of the IEC column are 6 min wide. The time between the dead volume and the last-eluting peak is 100 min. This equals a peak capacity of 16 in the first dimension. Returning to Figure 3a, the peak width is about 5 s; dividing the available elution time of 160 s (excluding the dead time) by this, the peak capacity is 32 in the second dimension. Multiplying 16 by 32 produces a chromatographic peak capacity of 512. The mass spectrometer also has inherent peak capacity, conservatively taken to be 5, because it can identify at least this number of components while scanning a 1000 m/z range. This makes the entire LC/LC/MS system's peak capacity greater than 2500.

The system is tested with a sample of pharmaceutical interest, a bacterial cell lysate often used to overexpress proteins for work in screening and structural studies. In this case, the chromatographic conditions are very similar to those used in the run of standards, only now the ion-exchange gradient begins at 14% instead of 20%. The UV absorbance data from the run of the *E. coli* cell lysate are shown in Figure 6. The extracted UV and ion current traces at 37.5 min make up Figure 7. The peak at 110 s in this RPLC run has the mass spectrum shown in Figure 8a, with its reconstructed molecular weight shown as 40 702 in Figure 8b. A search of the Swiss-Prot database shows 38 proteins from *E. coli* within 2% of this molecular weight but only two proteins within 0.2%, GCPE protein (P27433) and hydrogenase-1 small-chain precursor (P19928).

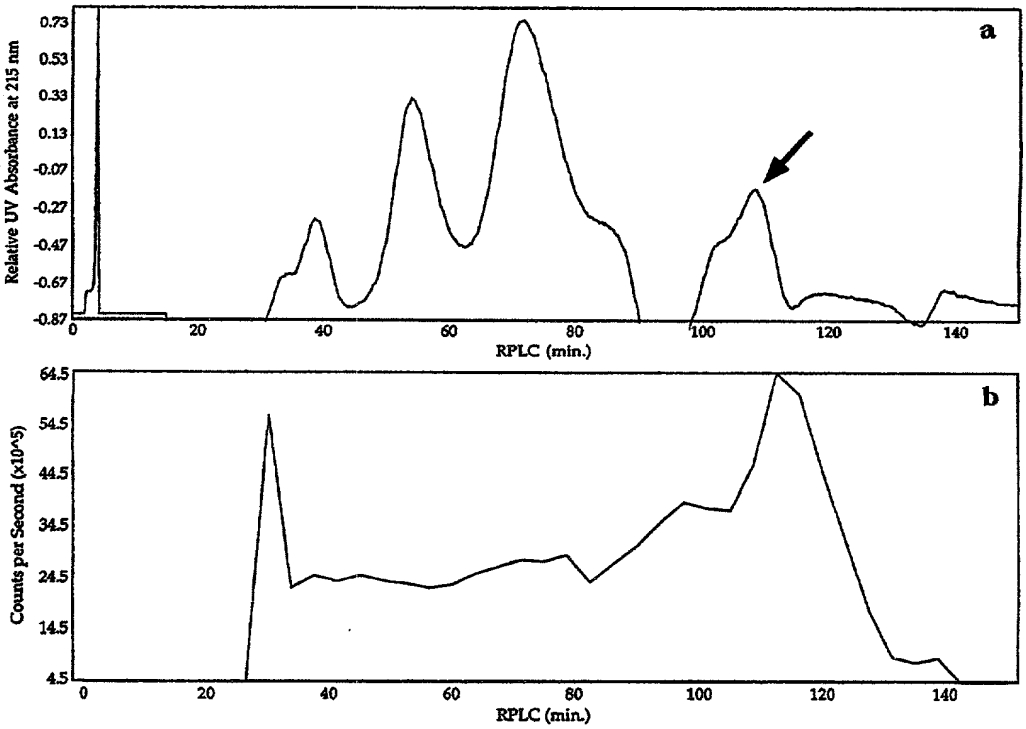

Figure 7. (a) UV absorption data from RPLC chromatogram extracted from 37.5 min of Figure 6. (b) Corresponding total ion current chromatogram.

DISCUSSION

A comprehensive 2D system without mass spectrometry can identify sample components on the basis of retention time. Of course, this is even more rigorous than a one-dimensional system because of the unlikely possibility that two components will have the same retention time in two orthogonal modes of separation. However, for complex samples that are uncharacterized, the addition of mass spectrometry as a detector to the system makes the system very capable of quickly screening for the major components, as can be seen in the run of *E. coli* lysate (Figure 7). At the end of the run, an analyte's retention times in two modes of chromatography, as well as its molecular weight within an accuracy of 0.2%, are known.

Compared to 2D polyacrylamide gel electrophoresis, this LC/LC systems pales with regard to peak capacity (>5000) and sensitivity (femtomole). However, to get a more precise molecular weight than that provided by the sodium dodecyl sulfate dimension, or to obtain the N-terminal sequence, a spot must be excised from the gel. This sample needs to be separated from the matrix, the SDS, and any stain used to develop the gel. These steps require a fair amount of operator effort and can introduce sample contamination and loss. The LC/LC/MS system described here, like the LC techniques it incorporates, is clearly faster and less prone to sample loss than 2D-PAGE. Additionally, the proteins can be recovered intact because of the 10:1 flow-split before the mass spectrometer. This allows for off-line proteolysis, Edman sequencing, or amino acid analysis.

Ion-exchange and reversed-phase modes of chromatography are used because they offer high-resolution separations of proteins. They are placed in such an order for two reasons. First, because of the necessity to run the second dimension quickly in order to minimize the recombination of components eluting off the first dimension, a stationary phase with a high optimum flow rate must be used. Only such reversed-phase materials are readily available and offer a large body of knowledge about their performance at high flow rates. Second, in order to couple to the mass spectrometer, the mobile phase of the second dimension must be amenable to electrospray ionization, again suggesting RPLC. These chromatographic modes are implemented on a microcolumn scale to improve efficiency as well as to reduce sample dilution for the electrospray ionization mass spectrometry, a concentration-sensitive technique.

Another way to look at the utility of this system is to recognize its ability to desalt the proteins on-line. By using a system such as this, established ion-exchange methods of protein separations can now be used as the basis for an LC–MS system, instead of having to discard those methods in favor of reversed-phase chromatography. A simple step gradient could be used in the second dimension if on-line desalting was the only goal. Since the cation-exchange chromatography is currently run at or below the optimum linear velocity in order to "broaden" the peaks, a step gradient would allow the first dimension to be run at higher linear velocities, reducing the time required to complete an experiment.

The UV trace and the ion current data both respond well to all 10 components in the standard mixture, but even the two weakest, α- and β-Hb, still show acceptable signals. Occasionally, a protein signal will be particularly broad and thus dilute. This often means that the ion current trace shows very little evidence of a peak. By having the UV detector in series, such a dilute peak can often be located from the absorbance data, allowing the

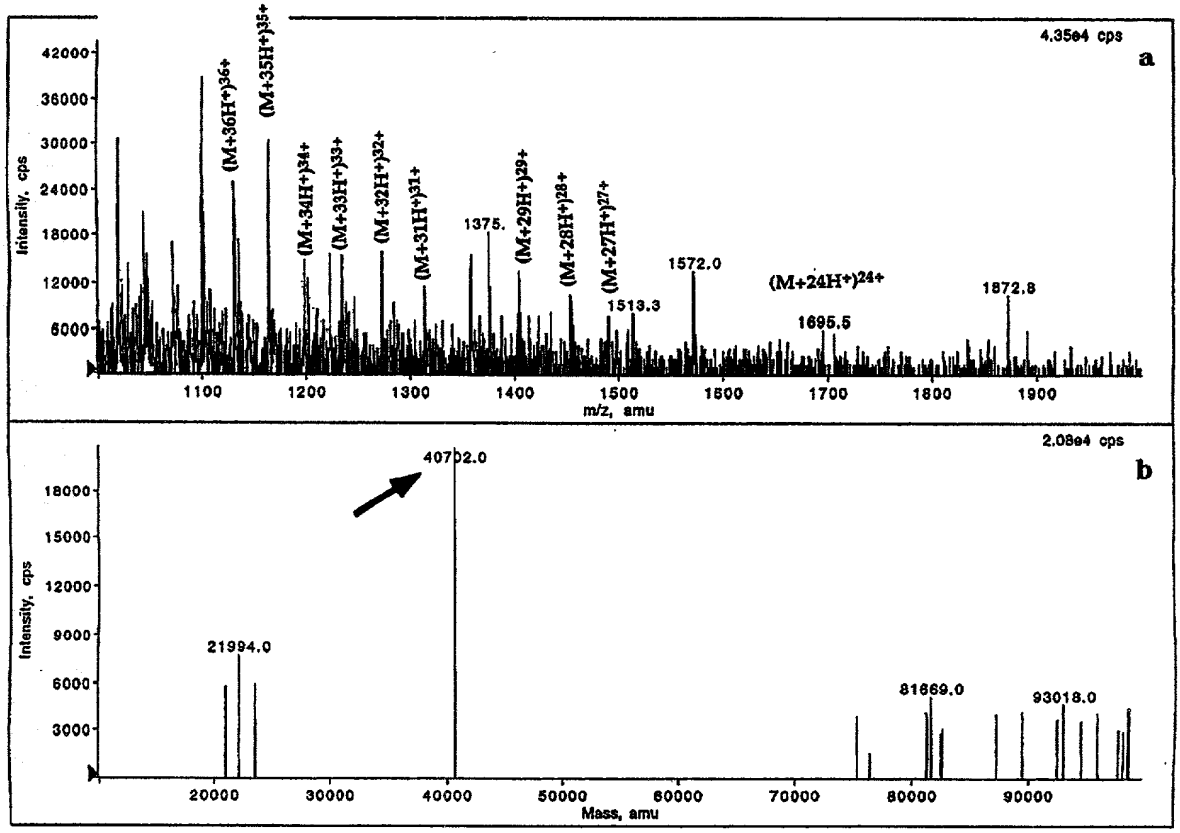

Figure 8. (a) Mass spectrum from peak at 110 s of Figure 7. (b) Corresponding Hypermass reconstruction of charge enve lope.

operator to target a certain region of mass spectral data in order to elucidate a charge envelope. Should the component not yield a usable mass spectrum, there exists another option. With the system described in this report, in lieu of one utilizing capillary electrophoresis for the second dimension, it is possible to collect fractions for off-line analysis.[21] All that is necessary is a fraction collector after the 10:1 flow split and before the mass spectrometer.

Numerous possibilities exist to improve this system. Perhaps the easiest is to increase the column length in the first dimension in order to increase efficiency. Unfortunately, this results in an increase in analysis time. A more efficient second dimension using a high flow rate stationary phase based on 5 instead of 10 μm particles would also be desirable. Not only would this increase the peak capacity and resolving power of the system, but it would also enhance the sensitivity of the system by making the peaks sharper and less dilute. Small increases in either dimension result in large overall gains in peak capacity, again because of the multiplicative effect of combining two systems in a comprehensive manner.

CONCLUSIONS

A comprehensive LC/LC system has been coupled to electrospray mass spectrometry in order to determine the molecular weights of proteins on-line. An entire mixture is separated in two orthogonal dimensions, without any prior knowledge of the individual components, e.g., p*I*, molecular weight, hydrophobicity, affinity. By coupling electrospray ionization mass spectrometry to an efficient chromatography system, a total peak capacity of over 2500 is obtained. The mass spectrometer can be presented with as little as 3.2 pmol of analyte and still obtain an accurate molecular weight. The use of reversed-phase chromatography allows the concentrated salt and urea ion-exchange mobile phase to be coupled directly to the mass spectrometer.

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